CD3-ε Overexpressed in Prothymocytes Acts as an Oncogene

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ABSTRACT

Background: Upon engagement of the T cell receptor for antigen, its associated CD3 proteins recruit signal transduction molecules, which in turn regulate T lymphocyte proliferation, apoptosis, and thymocyte development. Because some signal transducing molecules recruited by CD3-ε, i.e., p56lrk and p59fcr, are oncogenic and since we previously found that overexpression of CD3-ε transgenes causes a block in T lymphocyte and NK cell development, we tested the hypothesis that aberrant CD3-ε signaling leads both to abnormal T lymphocyte death and lymphomagenesis.

Materials and Methods: Ten independently derived transgenic mouse lines were generated with four different genomic CD3-ε constructs. Mice either homozygous or hemizygous for each transgene were analyzed for an arrest in T lymphocyte development and for the occurrence of T cell lymphomas.

Results: Aggressive clonal T cell lymphomas developed at very high frequencies in seven mouse lines with intermediate levels of copies of CD3-ε derived transgenes. However, these lymphomas were not found when high copy numbers of CD3-ε transgenes caused a complete block in early thymic development or when a transgene was used in which the exons coding for the CD3-ε protein were deleted. Analyses of a series of double mutant mice, tgCD3-ε × RAG-2null, indicated that lymphomagenesis was initiated in lineage-committed prothymocytes, i.e., before rearrangement of the T cell receptor genes. In addition, the transgene coding for the CD3-ε cytoplasmic domain and its transmembrane region induced a T cell differentiation signal in premalignant tgCD3-ε × RAG-2null mice.

Conclusion: The nonenzymatic CD3-ε protein acted as a potent oncogene when overexpressed early in T lymphocyte development. Lymphomagenesis was dependent on signal transduction events initiated by the cytoplasmic domain of CD3-ε.

INTRODUCTION

Tumorigenesis involves perturbation of multiple signal transduction cascades resulting in a profound disturbance of normal control of cell cycling, cell growth, and/or cell death (1–3). Whereas mutated oncogenes and tumor suppressor genes play a pivotal role in these processes (4–9), adapters of signal transduction pathways can also be tumorigenic (10–15). To investigate whether a surface receptor–associated protein that functions primarily by recruiting signal transduction molecules could also be tumorigenic, we analyzed large numbers of independently derived CD3-ε transgenic mice for spontaneous development of T cell tumors. CD3-ε is a T cell receptor (TCR)–associated membrane protein that plays a role in TCR/CD3 complex assembly and signal transduction (16–20). Upon engagement of the T cell receptor with antigen, CD3-ε directed signal transduction pathways are
initiated that regulate T cell proliferation, apoptosis, and thymocyte development (21–25). Because some signal transducing molecules recruited by CD3-ε, i.e., p56lck and p59fyn, are oncogenic (26,27), a disturbance of CD3-ε signaling could potentially lead to abnormal T lymphocyte death and lymphomagenesis.

As previously reported in a number of independently derived homozygous CD3-ε transgenic mice (i.e., tge26+/+), a very early block in thymocyte and natural killer (NK) cell development is observed. This block in development is caused by overexpression of the CD3-ε proteins derived from the transgenes that are regulated by their own cis-regulatory elements (28,29). Here we demonstrate that in hemizygous transgenic mouse lines, overexpression of CD3-ε resulted in an extremely high incidence of very aggressive T cell lymphomas. The lymphomas were observed in seven mouse lines carrying relatively high copy numbers of transgenes encoding CD3-ε-derived proteins with the capacity to recruit signal transduction pathways. However, lymphomas were not observed in transgenic lines in which CD3-ε expression was low or absent. These data indicate that a small nonenzymatic protein with a 55 amino acid cytoplasmic tail is an oncogene that can induce lymphomas in T lymphocyte precursors.

MATERIALS AND METHODS

Mice

Generation and screening of transgenic mice carrying all DNA constructs were carried out as described previously (28,29). For reasons of simplicity, we renamed the constructs in this report: pL12 as ε, pL12Δ1 as εΔ1, pL12Δ2 as εΔ2, and pL16 as εm (see ref. 28,29). RAG-2null mice were obtained from GenPharm International (Palo Alto, CA). lcknull mice were kindly provided by Dr. T.W. Mak. Double mutant RAG-2null × tge26, RAG-2null × tgeΔ1, and lcknull (ref. 30) × tge26 mice were obtained by breeding. The mice were housed in virus antibody–free (V.A.F.) conditions at the Animal Research Facility of Beth Israel Deaconess Medical Center.

Detection of Thymic Lymphomas

The frequency of lymphomas is a summary of mice with thymomas when sacrificed at 8 months of age and mice that died of lymphomas during that 8-month period, divided by the total number of mice monitored (40 or more unless otherwise stated). The frequency is an underestimation because the thymomas were scored by visual inspection of the thymuses and not by pathologic examination.

Flow Cytometry

Flow cytometric analysis of thymocytes and tumor cells for surface antigen expression was per- formed by three-color analysis as described previously (29).

Antibody Treatment of RAG-2null mice

Antibody treatment of RAG-2null mice was performed as described previously (31). Briefly, young RAG-2null mice (8 to 20 days after birth) were injected i.p. with 10 μg of anti-CD3ε mAb per gram body weight, and sacrificed for analysis 4 weeks later.

Histology

Tissues were frozen at −20°C and mounted for cryostat sectioning. Sections 5 μm thick were fixed in 4% paraformaldehyde, phosphate-buffered saline (PBS) (pH 7.4). The sections were stained with hematoxylin and eosin, or used for immunohistochemistry as described below. The sections were blocked with 2% normal rat serum, PBS (pH 7.4) for 20 min, stained with biotinylated primary antibodies for 1 hr, followed by ABC reagent (Vector Labs, Burlington, CA) for 45 min. The sections were counterstained with hematoxylin, dehydrated, and mounted. Biotinylated rat anti-mouse Thy-1.2 antibody was purchased from PharMingen (San Diego, CA).

RESULTS

High Incidence of T cell Lymphomas in Hemizygous tge26 mice

When 40 homozygous and hemizygous tge26 mice were monitored over a period of 1 year, almost all hemizygous tge26+−/− mice died of T cell lymphomas during that time period. The earliest tumor-induced death occurred at 3 months of age and the lymphomas resulted in 85% mortality by 8 months in these animals (Fig. 1, Table 1). In contrast, no tumors were detectable in homozygous tge26+−/− mice (Fig. 1), which is
consistent with the fact that tge26+/+ mice did not develop any T lymphocytes. The lymphomas observed in tge26+/- mice were always found first in the thymus, with the enlargement of one lobe visible from 8 weeks of age and older. In the later stages of the disease, the enlarged thymuses were up to 20 times their respective normal sizes, and enlarged spleens and lymph nodes were also frequently observed (Fig. 2A). Histological studies of these animals revealed metastases of the lymphomas in the trachea, lungs, liver, kidney, testis, and brain (Fig. 2B, and data not shown).

Flow cytometric analyses revealed that the thymic lymphomas were of T cell origin representing different stages of thymocyte development, the majority being CD4+8+ (Table 2). Southern blotting with a TCR-β probe indicated that the tumors were generally clonal, as 28 of 39 tumors (72%) had one or two rearranged TCR-β bands (data not shown, see Discussion). Upon transfer of these tumor cells into immunodeficient animals, i.e., nude, RAG-2null, and homozygous tge26 mice, or in syngeneic wild-type mice, death occurred within 3 to 4 weeks. Stable cell lines could be derived from approximately 1/6 of the tumors by in vitro tissue culture of the tumor cells without added growth factors. Generally, these cell lines resembled their parental tumors phenotypically (data not shown). Together, these observations support the notion that CD3-ε transgenes were involved in the generation of T lymphomas that were initiated early in T lymphocyte development.

![Graph of survival curve for tge26+/+ and tge26+/- mice.](image)

**Fig. 1.** Survival curve for tge26+/+ and tge26+/- mice.

Forty tge26+/+ and 40 tge26+/- mice were monitored for lymphoma-induced death. The data illustrate the high incidence of T cell lymphomas in tge26+/- mice.

<table>
<thead>
<tr>
<th>Transgenic Mouse Line</th>
<th>Copy No. of Transgene</th>
<th>Thymocyte (% of wt)</th>
<th>% of Mice with Tumors after 8 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>e</td>
<td>26</td>
<td>30-35</td>
<td>++: 1, +/−: 11</td>
</tr>
<tr>
<td>eΔ1</td>
<td>2978</td>
<td>40-45</td>
<td>++: 2, +/−: 16</td>
</tr>
<tr>
<td>2966L</td>
<td></td>
<td></td>
<td>++: wt, +/−: wt</td>
</tr>
<tr>
<td>eΔ2</td>
<td>2982</td>
<td>40-45</td>
<td>++: wt, +/−: wt</td>
</tr>
<tr>
<td>2994H</td>
<td>20-25</td>
<td></td>
<td>++: wt, +/−: wt</td>
</tr>
<tr>
<td>eε</td>
<td>2270</td>
<td>20-25</td>
<td>++: 5, +/−: 37</td>
</tr>
<tr>
<td>2279</td>
<td>10-15</td>
<td></td>
<td>++: 41, +/−: wt</td>
</tr>
</tbody>
</table>

Mice that were hemizygous for the same transgenes were only partially T cell deficient even though the homozygous counterparts had a complete developmental block. n.d., not determined; wt: wild type.

*Copy number refers to the copy number of transgenes in a hemizygous mouse (28).

*Total number of thymocytes as % of those in wild-type litter mates (28).

*The frequency was obtained as described in the Methods.
T cell Lymphomas Are Caused by Transgenic CD3-e Protein

To exclude the possibility that the T cell lymphomas in tge26+/- mice were induced by an insertion mutation of the transgene, nine additional CD3-e transgenic mouse lines (hemizygous and homozygous) were monitored for eight months (Table 1). These mice were generated with three different CD3-e genomic constructs: transgenes eΔ1 and eΔ2 representing the human CD3-e gene with two different deletions, and transgene e_m, representing a chimeric human-murine CD3-e gene, coding for the murine CD3-e protein (28,29). During a period of 8 months, high frequencies of T cell lymphomas were found in six of the nine lines. Timing of onset and phenotypes of these T lymphomas were similar to those in tge26+/- (Tables 1 and 2). Thus, a position effect of the CD3-e transgene in tumorigenesis was ruled out. Moreover, these data indicated that either the human or murine CD3-e protein could be oncogenic.

All seven lymphoma-prone transgenic lines had two important features in common: they carried relatively high copy numbers of different transgenes (>20X), and they were partially, but not completely, T cell deficient (Table 1). The frequency of tumors observed in several groups of hemizygous transgenic mice (e.g., tgeΔ1 or tge_m) increased with an increase in the number of transgene copies in those lines (Table 1). Importantly, in the same group of mice, the level of CD3-e protein expression in thymocytes also increased approximately with the increase in the number of the transgene copies (29). We conclude, therefore, that this transgene-induced lymphomagenesis was dependent upon overexpression of the CD3-e protein. This notion was strongly supported by the absence of tumors in animals with a wild-type phenotype owing to low transgene copy numbers (e.g., tgeΔ1-2966L) and in animals with high numbers of copies of
TABLE 2. Frequency of major T cell lymphoma phenotypes in transgenic mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>% of Tumors with Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TN</td>
</tr>
<tr>
<td>26</td>
<td>7.1</td>
</tr>
<tr>
<td>2978</td>
<td>14.3</td>
</tr>
<tr>
<td>2966H</td>
<td>0</td>
</tr>
<tr>
<td>3021</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Phenotypes were determined by flow cytometric analyses of the thymomal cells. In case cells from one tumor had more than one phenotype, the phenotype representing the majority of the tumor cells was chosen as the phenotype of the tumor. This table summarizes the data from flow cytometric analyses of 70 tumors. TN: CD3-CD4-CD8-; CD4+CD8+ or CD4-CD8+; DP: CD4+CD8+. Flow cytometric analyses also indicated that all tumors (except one) were Thy-1+CD24+, 65% were CD44+, 30% were CD25+, and 90% were CD5+.

transgene eΔ2 which did not contain the majority of CD3-ε coding sequences (Table 1).

Whereas no tumors were found in homozygous mice with a complete block in T cell development, e.g., tge26+/+ and tgeΔ1-2978+/+, T cell tumors were found in homozygous mice with partial T cell development (Table 1). For example, the thymic cellularity of the homozygous tgeΔ1-2966H mice was on average 13% of that in wild-type litter mates, and 50% of these tgeΔ1-2966H+/+ mice developed T lymphomas in 8 months (Table 1). The frequency of tumors in the homozygous tgeΔ1-2966H mice was even higher than that of the hemizygous mice of the same strain (50% versus 15%; see Table 1). Since the levels of transgene expression in homozygous mice were higher than those in hemizygous mice (29, and data not shown), this observation also supported the notion that overexpression of CD3-ε was involved in tumorigenesis.

As shown in Table 1, high frequencies of lymphomas were observed in tgeΔ1-2978+/− and 2966H mice. In the eΔ1 construct, the region encoding the ectodomain of CD3-ε was deleted from the original human genomic fragment, resulting in a truncated CD3-ε polypeptide that consisted of the 55 amino acid cytoplasmic domain and its membrane-anchor. This result suggests that the oncogenic signal could be initiated by this small nonenzymatic polypeptide.

CD3-ε-induced Tumorigenesis Occurs in Lineage-committed Prothymocytes

CD3-ε-induced tumor cells isolated from the thymus often represented T lymphocytes that were more immature than tumor cells derived from the spleen or lymph nodes of the same mice (Fig. 3, and data not shown). This finding, together with the observation that lymphomas were always first detected in the thymuses of tumor-bearing animals, indicate that cell trans-

FIG. 3. Two-color fluorescent profiles of lymphocytes isolated from thymuses and lymph nodes of 5 tge26+/− mice.

These data show the differentiation of lymphocytes after cell transformation.
formation occurred in thymocytes. Since our previous experiments demonstrated that overexpression of the CD3-ε-derived transgenes began at or prior to Day 13 of gestation (29), i.e., in prothymocytes, we examined whether the lymphomas were initiated in prothymocytes. To this end, two of the CD3-ε transgenic lines were bred with RAG-2null mice that have a block in T cell development at the CD44-CD25-CD4-CD8- control point (32). Indeed, as shown in Table 3, T cell lymphomas were frequently found in RAG-2null × tge26+/− and RAG-2null × tgeΔ1-2966H+/− mice, but not in mice that were RAG-2null × tge26+/− or RAG-2null without the transgene. These results indicate that the CD3-ε-induced tumorigenesis occurred prior to the rearrangement and expression of the T cell receptor.

All tge/tgeΔ1 × RAG-2null lymphoma cells represented immature thymocytes, as they were surface CD3− (data not shown). Some tumors had a phenotype of Thy-1+CD44−CD25+CD4−CD8− (Fig. 4A, tumor #1, and data not shown), representing very immature thymocytes (33,34). Interestingly, most tumors from the double mutant mice were CD4+CD8+CD3− (Fig. 4A, tumor #2, and data not shown), suggesting that the CD3-ε transgene might have signaled by itself to cause a partial progression of the tumor cell past the CD44−CD25+CD4−CD8− control point (32).

**Signal Transduction by CD3-ε Cytoplasmic Tail in Double-negative Thymocytes**

Several investigators have shown that CD3-ε could be triggered in CD4−CD8− (DN) RAGnull thymocytes by in vivo treatment with anti-CD3-ε antibodies (23,31,35). The anti-CD3-ε effect on DN thymocytes from RAG-1null or RAG-2null mice can be measured in terms of thymocyte proliferation, induction of the CD4 and CD8α and CD8β genes, as well as the abrogation of RAG-1 or RAG-2 transcription (23,36). To examine whether the transgene coding for the CD3-ε cytoplasmic tail could initiate signal transduction pathways in lineage-committed pretumor thymocytes, double mutant mice, i.e., RAG-2null × tge26+/− and RAG-2null × tgeΔ1-2966H+/−, were analyzed. In young (4 to 6 weeks) RAG-2null × tgeΔ1-2966H+/− mice, the transgene in-

### Table 3. Expression of CD3-ε-derived transgenes induces transition from DN to DP thymocytes in young RAG-2null and lcknull mice and lymphomagenesis in older mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total no. of thymocytesa × 10^6</th>
<th>% of Thymocytesb</th>
<th>% of Mice with Lymphomas at 4-8 monthsc</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAG-2null</td>
<td>5.6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>RAG-2null × tge26+/−d</td>
<td>0.3</td>
<td>5 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>RAG-2null × tgeΔ1+/+e</td>
<td>1.4</td>
<td>45 ± 26</td>
<td>55 ± 26</td>
</tr>
<tr>
<td>RAG-2null × tgeΔ1+/−</td>
<td>3.6</td>
<td>19 ± 14</td>
<td>81 ± 14</td>
</tr>
<tr>
<td>lcknull</td>
<td>9.4</td>
<td>5 ± 5</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>lcknull × tge26+/−</td>
<td>13.4</td>
<td>34 ± 16</td>
<td>66 ± 16</td>
</tr>
<tr>
<td>lcknull × tge26+/−d</td>
<td>1.4</td>
<td>11 ± 10</td>
<td>82 ± 10</td>
</tr>
<tr>
<td>lcknull × tge26+/−+d</td>
<td>0.8</td>
<td>5 ± 3</td>
<td>0</td>
</tr>
</tbody>
</table>

"Total number of thymocytes is the average of numbers from 4 to 10 mice.

"Phenotype of the thymocytes was determined by three-color flow cytometry. The conversion from DN to DP was probably going through a CD8 SP stage, as 5 to 33% of thymocytes were CD4+CD8+ cells in approximately 30% of the mice. For simplicity, these cells were categorized as DP cells in this table.

"The frequency was determined as described in Materials and Methods. The number of mice that died of lymphomas and the total number of mice monitored are indicated in parentheses. n.d., not determined.

"In RAGnull × tge26+/− and lcknull × tge26+/+ mice, the majority of thymocytes were Thy-1− B cells (29).

"tgΔ1 = tgeΔ1-2966H."
These observations indicate that overexpression of the CD3-ε cytoplasmic domain in prethymocytes mimicked the effects of anti-CD3-ε activation.

Previously, we showed that in RAG-1<sup>−/−</sup> mice, transition from DN to DP thymocytes can be only partially induced by anti-CD3 activation, which suggests that lck is an important element in signal transduction through the TCR/CD3 complex during the early stages of T cell development (23). However, lck<sup>−/−</sup> x tge26<sup>−/−</sup> double mutant mice developed lymphomas at a high frequency (Table 3 and Figures 4 and 5). Taken together, these analyses on double mutant mice shown in Table 3 and Figures 4 and 5 demonstrate that the cytoplasmic domain of CD3-ε could signal in DN thymocytes and that lck was not the only protein kinase involved in this signaling.

**DISCUSSION**

In this report we have shown that CD3-ε could function as an oncogene, since overexpression of the CD3-ε protein in prothymocytes was essential for the induction of T cell tumors. This conclusion is based on the following evidence: (1) tumors were observed in transgenic mice ex-
pressing functional CD3-ε-derived proteins, but not in transgenic mice made with a genomic construct that did not have any protein expression (εαΔ2); (2) the frequency of tumor incidence increased with an increase in the copy number of transgenes; (3) the level of transgene expression in thymocytes and peripheral T cells of these mice also increased with the copy number of transgenes (29); and (4) transgenes were overexpressed in immature thymocytes (from embryonic day 13 onwards) in mice carrying high copy numbers (29). By contrast, mice overexpressing human CD3-δ do not have an arrest in T lymphocyte development, nor do they develop lymphomas (29; B. Wang et al., unpublished data).

Overexpression of CD3-ε-induced cell transformation may be initiated in very immature thymocytes, for instance, in Thy-1⁺CD44⁺CD25⁻ thymocytes, since high frequencies of T cell lymphomas were observed in double mutant RAG-2null × tge26⁺/⁻ and RAG-2null × tgeΔ1-2966H⁺/⁻ mice. The transformed prothymocytes could continue to differentiate to give more mature phenotypes. Since tumors isolated from the transgenic mice (on RAG-2⁺/+ background) were generally clonal (one or two rearranged TCR-β genes on Southern blots), an unknown secondary event(s) may occur in the transformed. TCR-rearranged thymocytes, leading to the formation of clonal lymphomas. This notion is consistent with the theory of multistep tumorigenesis (2). One may predict that if the secondary event(s) occurs in prothymocytes prior to TCR gene rearrangement, lymphoma cells derived from one progenitor cell could continue to differentiate to display polyclonal TCR gene rearrangements. Indeed, no dominant rearranged TCR-β band(s) was detected in 28% of the lymphomas (11 of 39 examined) from the transgenic mice on a RAG-2⁺/+ background. It is noteworthy that timing and clonality of T cell lymphomagenesis in tge26⁺/⁻ mice were similar to those found in transgenic mice overexpressing lck, in transgenic mice expressing the chimeric homeobox gene E2A-PBX1 (37), and in hemizygous Ikaros mutant mice (38). However, there are some differences between these systems. We demonstrated that lck is not essential for CD3-ε induced T cell lymphomas, because both CD3⁺ and CD3⁻ tumor cells were observed in the double mutant lcknull × tge26⁺/⁻ mice. In contrast, only CD3⁻ tumors were found in the transgenic mice overexpressing lck (26). In hemizygous Ikaros mutant mice, T cell lymphomagenesis is preceded by a lymphoproliferation in thymocytes and peripheral T cells (38). This phenomenon was not observed in thymocytes and peripheral T cells derived from prelymphoma tge26⁺/⁻ mice.

We propose that overexpression of CD3-ε generates a signal to induce cell transformation, and we further postulate that the signal is related to normal signal transduction through CD3-ε. This hypothesis is supported by the observation that overexpression of CD3-ε mimics the effects induced by anti-CD3-ε treatment of thymocytes in vivo (23,31). For instance, overexpression of CD3-ε in thymocytes prevents intracellular TCR-β expression, induces apoptosis (29; D. Zheng et al., unpublished observations), and induces a transition from DN to DP in RAG-2null mice. Moreover, this hypothesis is consistent with earlier reports suggesting that murine leukemia virus–induced leukemogenesis is mediated through a signal(s) triggered by the binding of the virus with T cell receptors (39,40). Signal transduction through CD3-ε involves lck and other c-src-like kinases (20,41,42). Although lck is dispensable for CD3-ε-induced T cell lymphomas, it is still conceivable that induction of T cell tumorigenesis by CD3-ε and lck share common downstream pathways. Importantly, unlike lck, the 55 amino acid cytoplasmic tail of CD3-ε is not a protein tyrosine kinase, nor does it have any known enzymatic activity. The role of CD3-ε in oncogenesis may therefore lie in its function as a provider of docking sites for kinases and other signal transduction enzymes. There may be some similarity with nonenzymatic adapter proteins. This notion is consistent with recent observations that Shc, one of the nonenzymatic adapter proteins involved in a variety of signal transduction pathways, can induce cell transformation and survival in vitro culture systems (10–15). Nonenzymatic receptor proteins other than CD3-ε may therefore be potential oncoproteins under some circumstances.

ACKNOWLEDGMENTS

We thank Key Higgins for technical assistance, and Drs. Clyde Dawe, Tom Benjamin and Geoffrey Cooper for a critical review of the manuscript. This work was supported by a grant from the National Institutes of Health (PO1-AI 35714).
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Communicated by I. Weissman. Accepted on October 10, 1996.